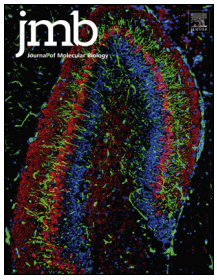
Review



Novel Insights into Cell Entry of Emerging Human Pathogenic Arenaviruses

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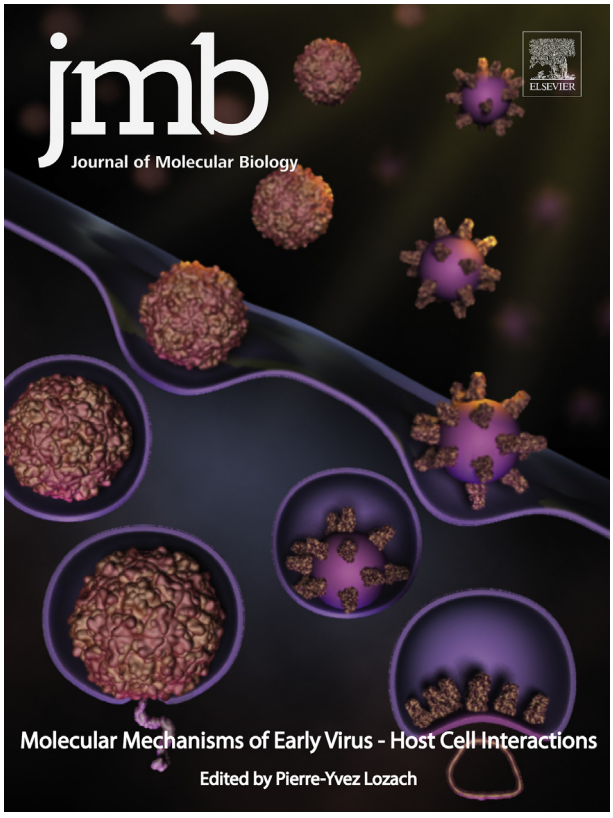
https://doi.org/10.1016/j.jmb.2018.04.026
Edited by P-Y Lozach

Abstract

Viral hemorrhagic fevers caused by emerging RNA viruses of the Arenavirus family are among the most devastating human diseases. Climate change, global trade, and increasing urbanization promote the emergence and re-emergence of these human pathogenic viruses. Emerging pathogenic arenaviruses are of zoonotic origin and reservoir-to-human transmission is crucial for spillover into human populations. Host cell attachment and entry are the first and most fundamental steps of every virus infection and represent major barriers for zoonotic transmission. During host cell invasion, viruses critically depend on cellular factors, including receptors, co-receptors, and regulatory proteins of endocytosis. An in-depth understanding of the complex interaction of a virus with cellular factors implicated in host cell entry is therefore crucial to predict the risk of zoonotic transmission, define the tissue tropism, and assess disease potential. Over the past years, investigation of the molecular and cellular mechanisms underlying host cell invasion of human pathogenic arenaviruses uncovered remarkable viral strategies and provided novel insights into viral adaptation and virus–host co-evolution that will be covered in the present review.



Legend: *This article is part of the thematic collection Molecular Mechanisms of Early Virus–Host Cell Interactions edited by Pierre-Yves Lozach (U. Heidelberg). Artwork by Rachel Davidowitz, Freelance Scientific Illustrator.*

Emerging human pathogenic arenaviruses are a major threat for public health

The Arenaviruses are a large and diverse family of emerging enveloped negative strand viruses that includes several severe human pathogens [1]. The *Arenaviridae* family is composed of the genus Mammarenavirus and the genus Reptarenavirus [2]. Based on phylogenetic and serological data, the mammarenaviruses are divided into two major groups: the Old World (OW) and the New World (NW) complex. For simplicity, we will henceforth use the term "arenaviruses" synonymous for the entire family and members of the Mammarenavirus genus. The OW arenavirus lineage contains the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) with worldwide distribution. The infection of LCMV in the mouse represents one of the most powerful models in experimental virology and immunology [3]. LCMV is further a relevant human pathogen in pediatric and transplantation medicine [4,5]. The OW arenavirus Lassa (LASV) is the causative agent of a severe viral hemorrhagic fever (VHF) with high mortality in humans. Carried by the persistently infected reservoir rodent host *Mastomys natalensis*, LASV is endemic in large parts of Western Africa resulting in an estimated 300,000 infections per year with several thousand fatalities [6]. There is currently no licensed vaccine and therapeutic options are limited resulting in case-fatality rates of 15–30% in hospitalized patients [7], making LASV currently one of the most important emerging pathogens [8]. Lujo virus (LUJV) emerged in 2008 as a novel arenavirus associated with a cluster of fatal human VHF in Southern Africa and represents a distant relative of LASV and LCMV [9]. The index case acquired the virus by an unknown route and initiated a chain of inter-human transmission [9]. The index patient and three of the contacts died, representing a case-fatality rate of 80%.

The genetically more diverse family of NW arenaviruses is organized in four Clades, A, B, C, and D. Since the 1950s, the Clade B NW arenaviruses Junin (JUNV), Machupo (MACV), Guanarito (GTOV), Sabia virus (SABV), and Chapare virus (CHAV) have emerged and cause outbreaks of human VHF with high mortality [10,11]. All human pathogenic NW arenaviruses belong to Clade B, whereas viruses of Clades A and C have so far not been associated with human disease. The current phylogenetic diversity of arenaviruses is likely the result of long-term co-evolution between viruses and their host species, involving vertical and horizontal transfer of viruses within and between populations [12]. Due to their transmissibility via aerosol [13] and high lethality, hemorrhagic arenaviruses are considered Category A agents by the Centers of Disease Control [14].

Arenavirus structure, infection, and human disease

The structure and life cycle of arenaviruses are subject of excellent reviews and we will give only a short summary here. Arenaviruses are enveloped negative-strand RNA viruses, whose life cycle is confined to the cytoplasm [1] (Fig. 1). In electron microscopy, viral particles appear spherical to pleomorphic, with diameters of 50–300 nm. The arenavirus genome is composed of two RNA segments. A small (S) RNA segment (3.4 kb) encodes the envelope glycoprotein precursor (GPC) and the nucleoprotein (NP) using an ambisense coding strategy. The large (L) segment of 7.2 kb encodes the matrix protein (Z) as well as the viral RNA-dependent RNA polymerase (L) (Fig. 1). The GPC precursor undergoes processing by signal peptidases and subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) to yield an unusually stable signal peptide (SSP), the N-terminal GP1, and the transmembrane GP2 [15]. The mature virion GP spike of arenaviruses comprises SSP/GP1/GP2 heterotrimers that represent the functional units of virus attachment and entry [15–17]. The GP1 binds to cellular receptors, whereas GP2 mediates viral fusion and structurally resembles class I viral fusion proteins. As detailed below, arenaviruses attach to the target cell via suitable receptors, followed by receptor-mediated endocytosis. The virus is delivered to acidified late endosomes, where low pH triggers fusion by an unusual mechanism covered in detail by an excellent review [18]. By an unknown mechanism of "uncoating," arenaviruses release their ribonucleoproteins that comprised viral RNA, NP, and L into the cytosol. Viral transcription initiates at the incoming polymerase complex, resulting in expression of NP and L [19]. Newly synthesized NP assembles the viral replication–transcription complexes that are membrane-associated structures containing cellular lipids and proteins [20,21]. During the final stages of the arenavirus life cycle, progeny particles assemble and are released by budding from the plasma membrane [22].

All known human pathogenic arenaviruses are zoonotic pathogens and reservoir-to-human transmission represents a major route of human infection [6,10,23]. Zoonotic transmission of arenaviruses occurs mainly via inhalation of aerosolized rodent excreta, skin abrasions, and ingestion of contaminated food [6,24]. Human-to-human transmission can occur in nosocomial settings and is associated with high mortality [25]. Following initial multiplication at the site of entry, the virus enters the bloodstream and disseminates to the lymph nodes, spleen, and

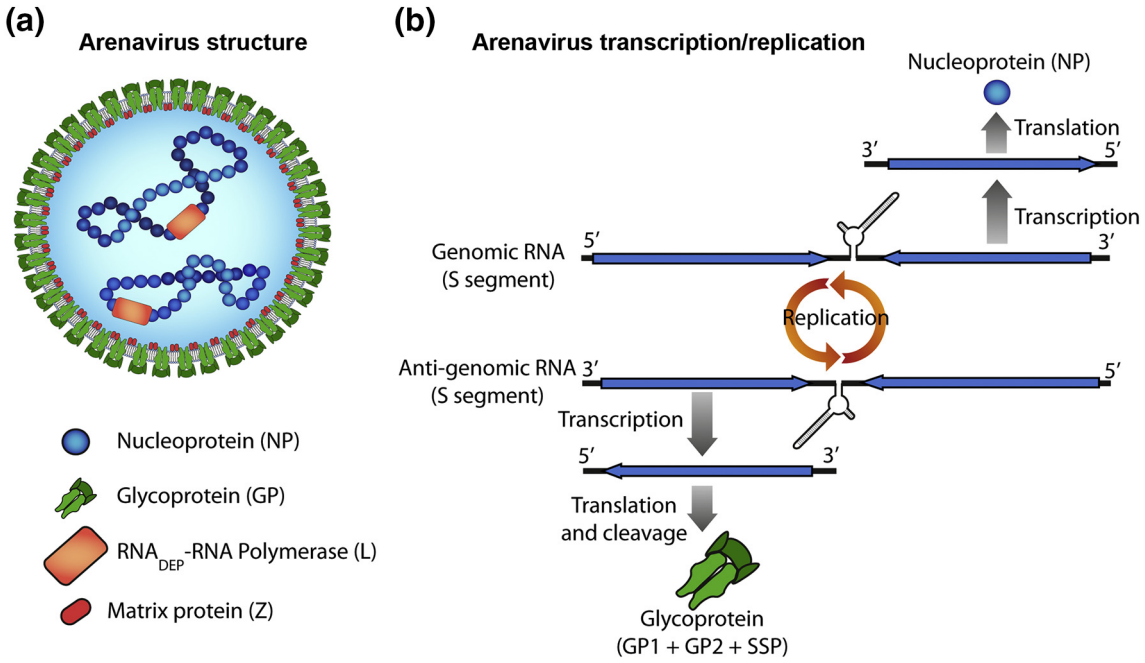


Fig. 1. Structure of arenavirus particles and their transcription/replication. (a) Structure of arenavirus particle. The surface is decorated with glycoprotein complexes composed of receptor-binding GP1, transmembrane GP2 subunits, and the SSP. The matrix protein Z is associated with the inner leaflet of the viral membrane envelope. Viral RNAs are associated with NP to form the ribonucleoparticles. The RNA-dependent RNA polymerase (L) is required for viral replication. (b) The viral genome contains two RNA segments, S and L with ambisense coding strategy. The genomic RNA represents the template for transcription and replication. For details, please see text.

liver [26]. Early targets of systemic infection are dendritic cells and macrophages, followed by infection of hepatocytes, vascular endothelial cells, and epithelia of the kidney and lung. Human arenavirus infection manifests initially with unspecific flu-like clinical signs and symptoms, including fever, weakness, and general malaise [6,10]. Severe infection is characterized by high serum viral load, edema formation, mucosal bleeding, and rapid deterioration. The pathophysiology of the fatal shock syndrome in arenavirus VHF is not well understood and may involve functional changes in the vascular endothelium, liver, adrenal gland, and other organs caused by the virus itself, as well as immunopathology [7]. A highly predictive factor for disease outcome is the viral load, indicating close competition between viral spread and replication and the patient's immune system [27]. Drugs targeting early steps of the viral life cycle can reduce multiplication and spread of the virus providing the patient's immune system a window of opportunity to develop a timely anti-viral immune response. An in-depth understanding of the molecular mechanisms underlying arenavirus entry is therefore of great importance to develop novel and efficacious strategies for anti-viral therapeutic intervention.

An old enemy: Lassa virus–receptor interaction and virus–host co-evolution

Among the arenaviruses, LASV represents the most prevalent human pathogen that currently threats over 200 million people in Western Africa. Recent genetic studies revealed that LASV is an ancient pathogen that invaded the human population >1000 years ago [23]. As a consequence, human LASV infection is widespread with seroprevalence reaching up to 50% in some regions [28]. At the community level, human LASV infection occurs mainly by reservoir-to-human transmission via inhalation or ingestion of contaminated rodent excreta [23]. Human-to-human transmission can cause explosive epidemics in nosocomial settings [25]. Epithelia lining the respiratory and digestive tract are likely early targets of LASV during zoonotic transmission. Productive entry of LASV into human epithelial cells involves the high affinity receptor dystroglycan (DG) discovered 20 years ago [29,30]. In mammals, DG is widely expressed in developing and adult tissues, where it provides a molecular link between the extracellular matrix (ECM) and the actin cytoskeleton. Apart from LASV, DG can serve as a receptor for most isolates of LCMV, the non-

pathogenic African arenaviruses Mopeia and Mobala, as well as Clade C NW arenaviruses [29,31].

During biosynthesis, the DG precursor undergoes auto-processing yielding the peripheral α-DG interacting with ECM proteins and the transmembrane β-DG that anchors the DG complex to the cytoskeleton. In mammals, α-DG is subject to remarkably complex and highly specific *O*-glycosylation involving >20 genes [32]. Functional glycosylation of α-DG starts with the biosynthesis of an unusual O-linked trisaccharide [*O*-Man-β1–4-GlcNAc-β1–3GalNAc] that undergoes phosphorylation at the *O*-mannosyl residue [33]. After the addition of a ribitol moiety, the dual-specific glycosyltransferase like-acetylglucosaminyltransferase (LARGE) attaches long chains of [Xyl-α1-GlcA-3-β1–3] co-polymers [34,35]. These negatively charged LARGE-derived sugar polymer called "matriglycan" bind to LG domains of ECM proteins via a lectin-type interaction [32,36,37]. Mutations in genes implicated in functional glycosylation of DG are relatively frequent in the human population and can manifest as neuromuscular diseases of varying degree, so-called "dystroglycanopathies" [38]. The unusual functional glycosylation of DG by LARGE is also crucial for arenavirus binding [39–41]. An elegant genetic screen used LASV as a "molecular probe" to decipher the complex functional glycosylation of DG and revealed that the virus strikingly mimics the binding mechanism of host-derived ECM proteins [42]. The recently solved high-resolution structures of the pre-fusion conformations of mature LASV and LCMV GP show that the trimeric virion spikes engage DG-linked matriglycan polymers via multiple contacts, resulting in high avidity binding with K_d <1 nM [16,17]. As a consequence, the virus is capable to successfully compete with ECM proteins like laminin [39,43–45] allowing efficient penetration of the ECM to engage cellular DG.

Most mammalian cells express the DG core protein. However, the functional glycosylation of α-DG is under tight developmental and tissue-specific control [36]. DG appears therefore as a "tunable" receptor [36], whose virus-binding affinity is determined by the complex spatiotemporal pattern of its functional glycosylation [39]. The high expression levels of functional DG in the epithelia of lung and digestive tract [46,47] suggest a role in zoonotic infection. Engagement of cellular DG by LASV in human epithelial cells induces receptor clustering and receptor-mediated signaling [48,49]. Virus-induced tyrosine phosphorylation of the cytosolic domain of β-DG promotes dissociation of the DG complex from the actin-based cytoskeleton, perhaps facilitating internalization [48]. The DG-mediated LASV entry into human epithelial cells involves an unusual mechanism of macropinocytosis that allows rapid internalization of the pathogen with only minimal perturbation of the host cell [30,50]. The remarkably high binding affinity of LASV to matriglycan at neutral pH suggests internalization of the virus–receptor complex. However, it is at present unclear whether DG acts as an authentic entry receptor or if yet unknown co-receptor(s) are involved in the entry process. After internalization, the virus passes through an unknown early endosomal compartment, followed by rapid delivery to late endosomes involving the endosomal sorting complexes required for transport [51–54].

Since high-affinity binding to DG is conserved among pathogenic and non-pathogenic arenaviruses [31,39,40], a direct role of the receptor in viral pathogenesis appears rather unlikely. It is, however, conceivable that functional glycosylation of DG may influence the efficiency of zoonotic transmission via aerosols or contaminated food. Long DG-linked matriglycan chains may reach above the glycocalix of epithelia, rapidly capturing free virus via a high-avidity lectin-type binding. Since LASV has been prevalent in Western Africa for >50 generations [23], the pathogen may have affected the genetics of human DG glycosylation in the region. Indeed, genome-wide association studies in human populations revealed positive selection for specific LARGE alleles in populations from Nigeria, where LASV has been endemic for many centuries [23,55,56]. Although the exact role of the selected LARGE alleles in LASV susceptibility of carrier individuals is not yet clear, these genetic data suggest a role of DG's post-translational modifications in virus–host co-evolution. It is further tempting to assume a role of the virus in the evolution of the remarkably complex genetics of DG glycosylation and the relatively high frequencies of gene defects in the human population.

NW arenaviruses: viral receptors and entry as determinants for human disease

In 2007, Radoshitzky and colleagues [57] identified human transferrin receptor (hTfR)-1 as the cellular receptor for the pathogenic Clade B NW arenaviruses JUNV, MACV, GTOV, and SABV. The conserved cargo receptor TfR1 plays a central role in cellular iron metabolism and is used by other viruses for cell entry [58]. The ubiquitous expression of TfR1 on cell types targeted by pathogenic Clade B viruses correlates well with the broad tissue tropism observed in human infection [10,59]. Binding of iron-loaded holo-transferrin to TfR1 located in coated pits triggers rapid endocytosis via clathrin-mediated endocytosis, followed by Rab5-dependent delivery to early endosomes [60]. In line with the use of hTfR1, JUNV enters via clathrin-mediated endocytosis and depends on Rab5 for productive entry [61,62].

Comparative studies of receptor use by pathogenic and non-pathogenic viruses showed that all viruses of Clade B use TfR1 orthologues, albeit with different species-specificities. Notably, the ability to use hTfR1 predicts the potential to cause VHF in man, illustrated by the recently discovered CHAV that was associated with a cluster of human fatal infections in Bolivia [11,63]. The reservoir hosts of JUNV, MACV, and GTOV are the rodent species *Calomys musculinus*, *Calomys callosus*, and *Zygodontomys brevicauda*, respectively. Only JUNV can use *C. musculinus* TfR1. JUNV and MACV, but not GTOV recognize the *C. callosus* orthologue, whereas GTOV is restricted to TfR1 of its reservoir *Z. brevicauda* [64], indicating species specificity. Interestingly, pathogenic NW arenaviruses do not cluster in a separate group of Clade B but occur in sub-lineages together with genetically closely related non-pathogenic viruses, for example, JUNV and MACV with Tacaribe virus (TACV) and GTOV with Amapari virus (AMPV) [12]. Although AMPV and TACV can use TfR1 orthologues derived from different mammalian species, including their reservoir hosts, the do not recognize the human TfR1 [65].

The cargo receptor TfR1 is a dimeric type II transmembrane protein, whose ectodomain is composed of a protease-like and a helical domain that bind transferrin, as well as an apical domain with unknown function. In contrast to OW arenaviruses, whose receptor binding closely mimics endogenous ligands, Clade B NW arenaviruses and transferrin use non-overlapping binding sites on hTfR1 [57]. Virus binding maps to a region within the apical domain containing a conserved tyrosine residue in position 211 in humans, cats, and reservoir rodents, but not rat and mouse. Remarkably, mutations of only six residues in murine TfR1 conferred binding to JUNV, MACV, and GTOV [64] and substitution of a single residue in hTfR1 allowed recognition of AMPV [65]. High-resolution structure analysis of the MACV GP1/hTfR1 complex revealed molecular details of receptor binding and corroborated the molecular determinants of species specificity [66,67]. These observations suggest that minor changes in the viral GPs, which may occur accidentally or due to selective pressure, could enable currently non-pathogenic Clade B viruses to infect human cells, allowing human transmission. The spatial separation of binding regions of virus and transferrin allows the host to acquire compensatory mutations that evade viral infection but retain the biological function of TfR1. Indeed, a recent study provided evidence for genetic divergence at the TfR1 locus in rodents driven by escape from viral infection with simultaneous preservation of transferrin binding [68]. The resulting genetic differences enforce species barriers to virus transmission, limiting spillover. From a therapeutic perspective, a monoclonal antibody targeting the apical domain of hTfR1 blocks entry of all known pathogenic Clade B NW arenaviruses, providing proof-of-concept [63].

Further evidence for a critical role of NW arenavirus entry as a determinant of virulence comes from recent studies on the JUNV vaccine Candid#1 currently used with success in high-risk populations in Argentina. This live-attenuated vaccine was developed by serial passages of the pathogenic JUNV strain XJ13 [69]. Reverse genetics approaches mapped attenuation of JUNV Candid#1 to the GPC gene, in particular the mutation F421I in the fusion-active GP2 [70,71]. The effect of the substitution F421I on JUNV GPC function seems rather complex. Initial structure–function analysis indicated effects of the F421I mutation on the overall stability and receptor binding of the mature JUNV Candid#1 GP spike [72]. A more recent study revealed an interesting link between the attenuating F471I mutation and the regulation of JUNV GP fusion by the SSP [73]. In sum, the data at hand indicate that receptor binding and cell entry are important determinants for disease potential in Clade B NW arenaviruses.

A new distant relative: receptor use and pathogenesis of LUJV

The South African LUJV represents the most recently emerged hemorrhagic arenavirus [9]. The reservoir of LUJV remains elusive and the virus has not re-appeared since the initial outbreak in 2008. The suspected airborne transmission and high case-fatality rate make LUJV a major public health concern. The identification of its cellular receptors and entry pathways is of high priority to assess the risk of zoonotic transmission and to gain insights into pathogenesis. Infectious LUJV was isolated from the blood of the second case and a reverse genetic system established, opening the avenue of structure–function studies [74]. Shortly after its discovery, it became clear that LUJV enters human cells independently of DG and hTfR1 [75], in line with the highly divergent sequence of LUJV GP1 [9]. A recent elegant genome-wide haploid screen for LUJV cell entry factors revealed a prominent gene cluster involved in heparan sulfate biosynthesis, which serves as an attachment factor for many viruses [76]. Another prominent hit was neuropilin (NRP)-2, a cell surface receptor for semaphorins. LUJV engages NRP2 at the cell surface with high affinity involving the N-terminal domain of the receptor [76]. Upon viral attachment to NRP2, the virus–receptor complex is rapidly internalized and enters the endosomal route, identifying NRP2 as an authentic entry receptor.

Despite the genetic distance, the clinical signs and symptoms of LUJV VHF overall resemble severe Lassa fever [77]. However, in contrast to Lassa

fever, LUJV VHF has a more abrupt onset and involves stronger coagulopathy, evidenced by disseminated intravascular coagulation, which is rare in Lassa fever [77]. Interestingly, NRP2 is highly expressed on microvascular endothelia [76], making these cells highly susceptible for LUJV. This strong tropism of LUJV for the microvasculature may explain, at least in part, the stronger coagulopathy observed in LUJV VHF [77]. Likewise, expression of NRP2 on alveolar macrophages may facilitate aerosol transmission of LUJV, suspected as a possible route of inter-human transmission [77].

Apoptotic mimicry in arenavirus hemorrhagic fever virus spillover and disease

Virus attachment to cellular receptors represents a major species barrier for zoonotic pathogens. Since virus–host co-evolution is driven mainly by long-term relationships of viruses with their reservoir hosts, there is no *a priori* selection pressure to evolve the capacity to recognize human receptors. An elegant strategy of infection that many viruses evolved consists of acquiring phosphatidylserine (PS) in their envelope during budding, and consequently be disguised as apoptotic bodies that can be engulfed by cells through clearance mechanisms [78,79]. Viral cell entry via this mechanism of "apoptotic mimicry" was first demonstrated by Mercer and Helenius for the poxvirus vaccinia [80]. Apoptotic mimicry is currently recognized as a major entry strategy used by a broad spectrum of viruses, including important emerging pathogens, such as Ebola, Dengue, West Nile, and Zika virus [81–86]. In live cells, PS is restricted to the inner leaflet of the plasma membrane due to the activity of "flippases" and "floppases" [87,88]. During budding, enveloped viruses incorporate patches of cell-derived membrane lipid bilayers into their virion particles. The lack of flippase and floppase activities in virions results in progressive exposure of PS on the external leaflet, where it can act as an "eat me" signal for specific PS receptors [89]. The major classes of cellular PS receptors currently implicated in viral entry via apoptotic mimicry are molecules of the T-cell immunoglobulin and mucin receptor (TIM) family, in particular TIM-1 and TIM-4 and receptor tyrosine kinases of the Tyro3/Axl/Mer (TAM) family [78,79]. TIM-1 and TIM-4 directly bind to PS exposed in the viral membrane via their globular head domain. Virus binding to TAM receptors requires the PS binding proteins Gas6 and protein S that are present in serum and provide a molecular bridge between TAM receptors and the virus [79,90,91].

Removal of apoptotic bodies by PS receptors is an evolutionary ancient and highly conserved mechanism that appeared with early multicellular lifeforms [92]. Apoptotic mimicry therefore allows viruses to break barriers between distantly related species, such as arthropods and humans. The PS receptors of the TIM and TAM families are widely expressed in many tissues, including epithelial cells lining the body's mucosal surfaces, vascular endothelial cells, hepatocytes, different classes of immune cells, kidney, nervous tissues, heart, and skeletal muscle [83,93–96], conferring broad tissue tropism. In addition to their role in removal of apoptotic cells, TAM receptors play a crucial role in the negative regulation of innate immune signaling, regulating the host cell's type interferon (IFN)-I response [92,97,98]. Engagement of TAM receptors by enveloped viruses downregulates innate immune signaling, blunting the IFN-I response, thus promoting viral replication at the post-entry level [78,79,99].

Several hemorrhagic fever viruses use apoptotic mimicry to enter human cells, including arenaviruses, filoviruses, and flaviviruses [79]. However, the specific contribution of apoptotic mimicry greatly varies. The TAM and TIM receptors can mediate cell entry of filoviruses and flaviviruses independently of the envelope GP and likely play an important role in human infection [83–86,100–103]. The role of apoptotic mimicry in human pathogenic arenavirus infection is less clear. As described above, LASV and other OW arenaviruses evolved to recognize the evolutionary highly conserved DG-linked matriglycan, which is abundant in in many human cell types. However, at later disease stages, LASV spreads systemically and the broad viral tropism does not always correlate with the expression of functional DG [7,104,105]. Adult hepatocytes are, for example, highly susceptible to LASV infection but essentially lack functional DG, suggesting the existence of alternative receptors [47,106]. Indeed, expression cloning identified the TAM receptors Axl and Tyro3, as well as the C-type lectins DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) and LSECtin as candidate alternative LASV receptors [107]. Based on their known expression patterns, DC-SIGN and LSECtin may contribute to LASV entry to specific cell types, such as dendritic cells. However, in contrast to Phleboviruses that use DC-SIGN as authentic entry receptor [108,109], the exact role of DC-SIGN in LASV entry is currently unclear [110].

Human cells targeted by LASV *in vivo* frequently co-express PS receptors like Axl with differentially glycosylated DG. The tissue-specific receptor use of LASV may therefore vary, depending on the functional glycosylation of DG as shown in our model in Fig. 2a [106,107]. Epithelial cells lining the respiratory and digestive tract express high levels of functional DG [46,47], which may act as a major receptor for LASV [39,40,42], contributing to zoonotic transmission. In cells lacking functional DG, PS receptors like Axl can contribute to LASV entry via

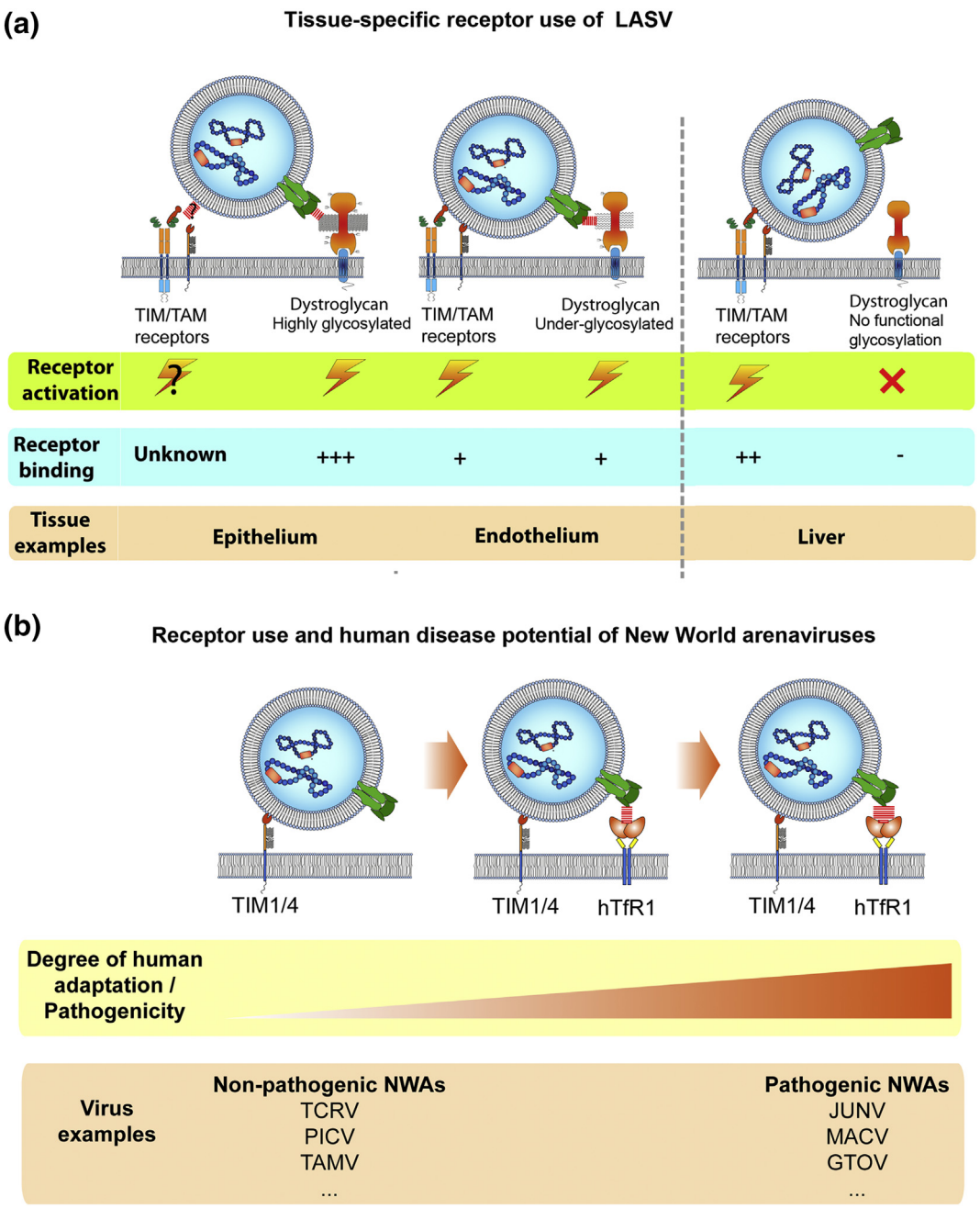


Fig. 2. The role of apoptotic mimicry in arenavirus infection. (a) Tissue-specific receptor use of LASV depends on the functional glycosylation of DG. In epithelial cells, highly glycosylated DG may act as a main receptor that undergoes virus-induced phosphorylation, promoting viral entry via macropinocytosis. Whether PS receptors can indirectly contribute to viral entry is currently unknown. In endothelial cells that co-express under-glycosylated DG, PS receptors can directly contribute to viral entry. In absence of functional DG, PS receptors may act as alternative LASV receptors. (b) Receptor use correlates with human adaptation and disease potential of NW arenaviruses. Infection of human cells by pathogenic NW arenaviruses is mediated by hTfR1 that only partially cooperates with PS receptors for efficient entry [100]. In contrast, non-pathogenic NW viruses infect human cells mainly via PS receptors with little contribution of hTfR1.

"apoptotic mimicry," similar to other enveloped emerging viruses [106,107]. Axl-dependent LASV entry requires virus-induced receptor activation and involves a pathway resembling macropinocytosis [106,107]. However, in contrast to flaviviruses, virus-induced activation of Axl seems not required for post-entry steps of OW arenavirus infection [106]. The OW arenaviruses can efficiently evade innate

immunity due to the potent IFN antagonist function of the viral NP and Z proteins, perhaps making attenuation of the IFN-I response obsolete [111–115]. Vascular endothelial represent important targets for LASV in late infection [116]. They express high level of Axl in combination with an underglycosylated form of DG that shows reduced virus-binding affinity [106]. Interestingly, both receptors contribute to productive LASV entry, in line with the high susceptibility [106,117] (Fig. 2a). So far, expression cloning revealed only Axl and Tyro3 as candidate PS receptors involved in LASV entry [107]. However, other import PS receptors, including, for example, molecules of the TIM family may contribute to LASV infection in specific cell types.

In contrast to LASV that can use PS receptors depending on the functional modification of its main receptor DG, the role of PS receptors in NW arenaviruses seems to correlate inversely with the extent of their adaptation to humans (Fig. 2b). Overexpression of TIM and TAM receptors in hTfR1-positive cells results in only mild increase of infection with pathogenic NW arenaviruses like JUNV and MACV, consistent with a role of hTfR1 as major entry receptor [100] (Fig. 2b). In contrast, TIM and TAM receptors can significantly contribute to entry of non-pathogenic viruses like TCRV and AMPV that cannot recognize hTfR1 into human cells [100] (Fig. 2b). The receptor binding data at hand suggest that pathogenic and non-pathogenic NW arenaviruses have undergone distinct degrees of human adaptation. Pathogenic viruses like JUNV are capable to hijack hTfR1 in cooperation with TAM/TIM, facilitating rodent-to-human transmission and viral spread within the human body, whereas non-pathogenic viruses like TACV may critically depend on TAM/TIM. The partial dependence of pathogenic NW viruses on TAM/TIM receptors (Fig. 2b) suggests that there may still be room for improvement regarding receptor-binding affinity, which may further increase virulence. However, increased binding to hTfR1 is not expected to affect TAM/TIM binding of pathogenic NW viruses (Fig. 2b). Currently, non-pathogenic NW viruses like TCRV and AMPV may use TAM/TIM receptors to break the species barrier between reservoir and humans. However, continued spillover may result in selection of viral variants capable to bind to hTfR1, which may affect transmissibility and virulence and is a concern from a public health perspective.

Receptor switches and endosomal entry factors: a novel paradigm for viral entry

Engagement of cell surface receptors by arenaviruses and filoviruses is followed by the internalization of the virus–receptor complex via endocytosis and delivery to acidified endosomes, where viral fusion occurs under low pH (<5.5). Based on their low fusion pH and endosomal escape kinetics, they are considered “late penetrating” viruses [118]. Pioneering work on cell entry of Ebola virus using independently haploid genetic screens and high-throughput small molecule screening identified the late endosomal host protein Niemann–Pick C1 (NPC1) as a crucial entry factor [119,120], providing a novel paradigm for viral entry. NPC-1 is a ubiquitously expressed multi-membrane-spanning protein localized in the limiting membrane of late endosomes and lysosomes, where it is involved in cholesterol trafficking [121,122]. At the level of late endosomes, EBOV GP undergoes processing by cathepsins, which unmasks a binding site for NPC-1, orchestrating endosomal delivery with engagement of NPC-1, which acts as a late endosomal intracellular receptor [119,123]. The endogenous activity of NPC-1 is not required for its role in EBOV entry, as NPC-1 mutants deficient in cholesterol trafficking are still able to bind EBOV GP [123] and to promote cellular infection [120,123]. Following binding, NPC-1 is involved in fusion triggering of EBOV GP, by approaching the viral and the endosomal membrane and allowing first lipid bilayers hemifusion and subsequently the fusion pore formation.

Performing haploid genetic screening, Jae *et al.* [124] identified the lysosome-associated membrane protein (LAMP)-1 as a late endosomal entry factor required by LASV in analogy to NPC-1 in EBOV. The acidic pH of the late endosome destabilizes the high-affinity interaction between LASV GP and DG, resulting in a “receptor switch” to LAMP1, which triggers fusion (Fig. 3). NCP-1 co-localizes with the EBOV entry receptor TIM-1 in the same endosomal/lysosomal compartments, suggesting a similar “receptor switch” at the level of the late endosome for filoviruses [103]. Structural analysis of LASV GP1 combined with functional studies revealed a stable “low pH conformation” of LASV GP1 containing a triad of histidine residues involved in LAMP1 binding [125]. Electron cryotomography on LASV GP spikes on viral particles confirmed irreversible conformational changes in GP1 induced by low pH [126]. Around pH 5.5, residue H230 within the histidine-triad on LASV GP1 undergoes protonation, which stabilizes the pre-fusion conformation, preventing fusion prior to binding to LAMP-1 [127]. Subsequent engagement of protonated GP1 with LAMP1 may neutralize the positive charge, promoting efficient fusion triggering at the proximity of the membrane [127] (Fig. 3). This indicates a remarkable role of the histidine-triad in orchestrating fusion activity of LASV GP with the timing and location of the receptor switch. Fusion triggering by LAMP1 allows LASV to exit from earlier, less acidic endosomal compartments, avoiding prolonged exposure to the hostile environment [128]. A critical role of LAMP1 is observed with DG- and Axl-dependent LASV entry

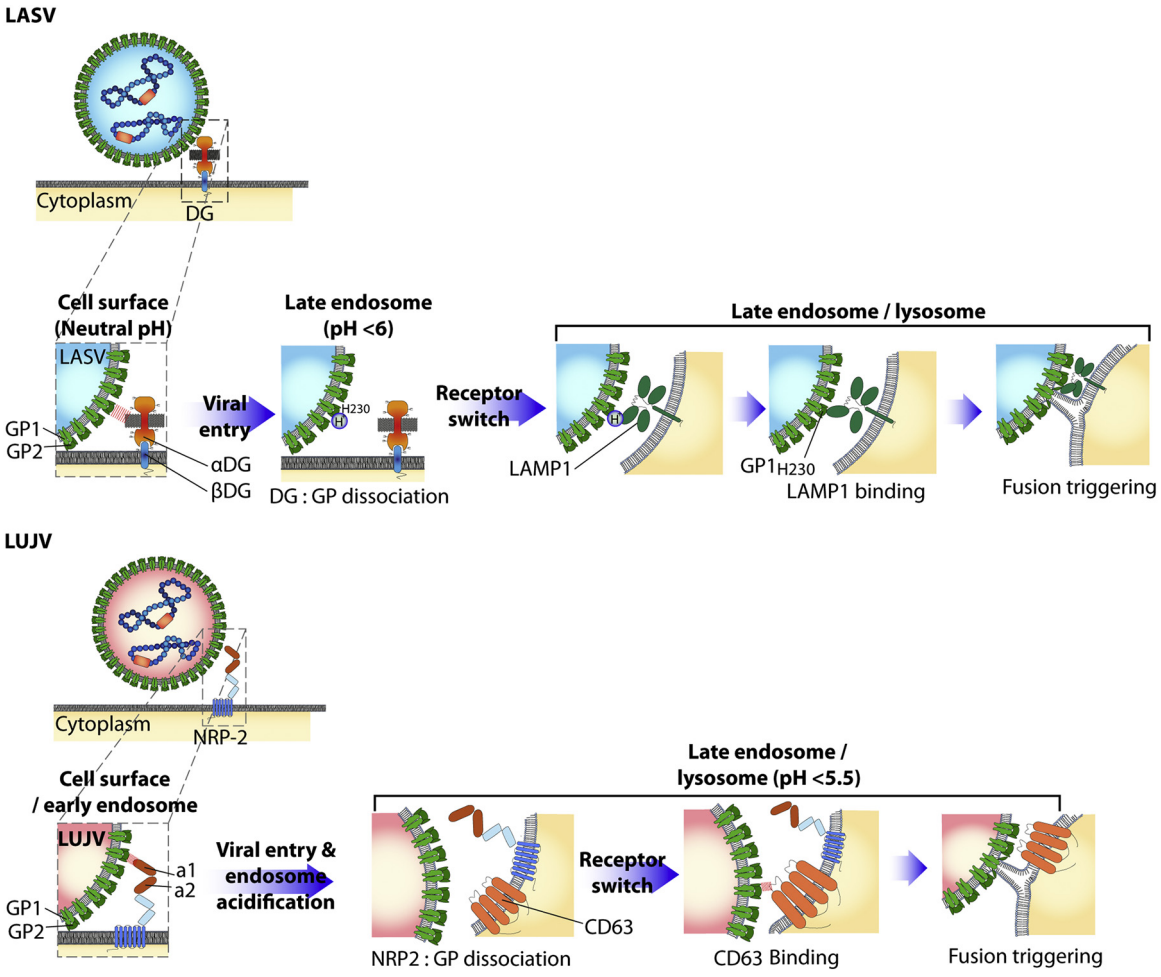


Fig. 3. Current model for the late endosomal "receptor switch" of arenaviruses. At the cell surface, LASV engages functional DG, followed by endocytosis. Progressive acidification of late endosomes induces a structural transition of LASV GP1, which dissociates from DG and adopts a low pH conformation displaying a histidine-triad. Protonation of residue H230 stabilizes the pre-fusion state of GP1, preventing premature fusion. Subsequent engagement of LAMP1 neutralizes the positive charge on H230 and triggers fusion with the limiting membrane of the late endosome/lysosome. The distantly related LUJV uses a similar strategy, involving cell surface binding and entry via NRP-2, followed by a late endosomal receptor switch to CD63, resulting in fusion triggering. For details, please see text.

[106,124], suggesting conversion of the entry pathways at similar late endosomal compartments. Interestingly, the capacity to hijack LAMP1 to facilitate late endosomal fusion triggering is unique for LASV and is not observed with other arenaviruses [129].

The above-mentioned haploid screen for LUJV entry factors revealed the tetraspannin protein CD63 as another prominent hit [76]. As NPC-1 and LAMP1, CD63 localizes to late endosomal compartments, suggesting an analogous role in viral fusion. Current evidence supports the notion of a similar late endosomal "receptor switch" for LUJV (Fig. 3). However, in contrast to the high affinity LAMP1 binding of LASV GP1 at low pH [124], the LUJV GP–CD63 interaction seems more transient [76]. In sum, the data at hand suggest that in analogy to the filovirus EBOV, the hemorrhagic OW arenaviruses LASV and LUJV evolved a similar *modus operandi* of late endosomal "receptor switch" to invade human cells. This represents a striking example of convergent evolution and offers promising novel targets for therapeutic intervention.

Perspectives and challenges

Recent developments on arenavirus entry provided novel insights into the complex mechanisms underlying this fundamental step of viral infection. At the same time, new questions arise. Several lines of evidence highlight the importance of DG's remarkably complex functional glycosylation for OW arenavirus receptor function. Accordingly, expression of

functional DG in many cell types is consistent with susceptibility [47]. However, skeletal muscle is rich in functional DG, but appears largely resistant to viral infection [104,130]. A recent study demonstrated that the block lies at the level of entry, indicating that expression of functional DG is not sufficient for productive infection [131]. A hallmark of DG is its complex "interactome" resulting in the formation of large supramolecular DG complexes [132] with tissue-specific composition that likely represent the actual "functional units" for virus entry. Pre-existing "steady-state" protein–protein interactions may affect DG's ability to function as a viral receptor. Cellular DG interaction partners may therefore act as tissue-specific susceptibility factors that can influence viral tropism and disease outcome. Moreover, engagement of cellular DG by the virus can induce signaling and may affect the molecular composition of the complex [48,49,106]. Indeed, virus–receptor binding can alter the steady-state pattern of protein–protein interactions in a dynamic manner, as recently illustrated in a ground-breaking proteomic study on hepatitis C virus entry [133,134]. Novel "shotgun" proteomics approaches specifically developed for the study of virus–receptor interactions provide new and powerful techniques to address these questions in the context of arenaviruses [133,134].

Viruses can actively induce cellular signaling as a "knock on the door" to promote entry and to "prime" the host cell to facilitate subsequent steps of infection. In order to send such "instructive signals" to the host cell, viruses may rely, at least in part, on signaling pathways pre-linked to receptor, co-receptor, and entry factors. Since viruses are inherently opportunistic, they also may be able to "create" their own signaling pathways by recruiting cellular factors according to their specific needs. Unbiased quantitative phosphoproteomics, recently successfully used to dissect the complex signaling induced by human immunodeficiency virus (HIV)-1 [135], opens the possibility to uncover "viral entry signaling networks" that underlie host cell invasion by pathogenic arenaviruses. Conserved candidate signaling pathways involved in productive entry of several relevant pathogens may allow the identification of promising targets for broadly active anti-viral drugs. Since signaling molecules like cellular kinases may represent well-characterized drug targets for therapeutic intervention against other human disorders, clinically approved drugs or drug candidates in advanced stages of development may be "re-purposed" to combat human pathogenic arenaviruses.

One of the most exciting recent discoveries in viral entry is the identification of late endosomal co-receptors, such as NPC-1 for Ebola virus, LAMP-1 for LASV, and CD63 for LUJV [76,119,124]. Excellent structural work, combined with functional studies, revealed the modus operandi of LAMP-1 in LASV entry, revealing the ability of the virus to precisely orchestrate endosomal transport with efficient fusion triggering [125,127,129]. The interesting parallels between the use of NPC-1 by the filovirus Ebola and LAMP-1/CD63 by pathogenic OW arenaviruses provide a novel paradigm in virus–host cell interaction probably relevant for other emerging viruses and virology at large. It will be very interesting to see if other arenaviruses, such as LCMV and the NW viruses or other emerging viruses, for example, Bunyaviruses and Coronoaviruses, evolved to hijack other, yet unknown late endosomal factors that facilitate productive entry and contribute to tissue tropism and disease potential.

Acknowledgments

The authors would like to apologize to all those colleagues whose excellent work could not be covered due to space limitations. This research was supported by Swiss National Science Foundation grants 310030_149746 and SINERGIA CRSII3_160780 to S.K., and funds to S.K. from the University of Lausanne.

Received 2 February 2018;
Received in revised form 19 April 2018;
Accepted 21 April 2018
Available online 27 April 2018

Keywords:
viral entry;
receptor;
arenavirus;
tropism;
zoonosis

Abbreviations used:
OW, Old World; LCMV, lymphocytic choriomeningitis virus; VHF, viral hemorrhagic fever; NW, New World; GPC, glycoprotein precursor; NP, nucleoprotein; SSP, stable signal peptide; DG, dystroglycan; ECM, extracellular matrix; PS, phosphatidylserine.

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